SUMMARY OF THE PhD THESIS

PERIODONTAL CHANGES IN SYSTEMIC CONTEXT IN CHILDREN AND TEENAGER

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„Lucrarea omului trebuie să fie ca un diamant,
să strălucească oricum ai intoarce-o”

Sfântul Luca al Crimeii - Chirurgul fără de arginți (1877-1961)
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A. STATE OF THE ART

In the past ten years, there was a considerable recognition of the link between clinical and pathogenic factors of periodontal disease/periodontitis and systemic alterations in general, including diabetes, revealing therefore new insights on understanding the pathogenesis of periodontal disease - PD, associated with various clinical applications. There is basically in the last decade a real redefinition of the inter-relationship between oral health and individuals particularities, reevaluating the considerable significance of periodontal homeostasis and functional integrity in the context of optimal functioning of the body.

Periodontal diseases/periodontitis are common oral alterations affecting people around the world for centuries, being chronic, painless disorders, which makes subjects rarely seek for professional assistance. Left untreated, these injuries ultimately lead to the destruction of dental appliance support units, being possible end up in partial or complete tooth loss (Armitage, 2004 Pihlstrom et al., 2005).

Periodontal medicine focuses on gathering evidence-based data which establish a significant relationship between periodontitis and systemic health (Williams & Offenbacher, 2000). The type of immune response developed by the host tissues confronted with periodontal pathogen is crucial, conditioning their resistance or susceptibility in the installation and running of clinical symptoms of periodontal disease.

Several inflammatory mediators such as cytokines, prostaglandins and matrix metalloproteinases are necessary in orchestrating immuno-inflammatory events which determine the pathogenesis of periodontal disease (Gemelli et al., 1997). The expression of these mediators is significantly regulated by various cell populations, from neutrophils (considered the first line of defense in case of aggression during periodontal alterations) to mononuclear cells and T lymphocytes (Nussbaum et al., 2011, Kolaczkowska & Kubes, 2013). The development of these concepts has enabled switching to a new paradigm, through worsening periodontitis complex character, whose expression involves an interaction between the microbial biofilm and the host's inflammatory immune response, ultimately responsible for modulating periodontal tissue homeostasis (Kornman, 2008).

Inter-relationship diabetes - periodontitis has been represented a constant preocupation of scientists for many years and, although some studies have shown no correlation between the two entities with common inflammatory pathogenic aspects, most studies have revealed the existence of a significant prevalence of alterations in periodontal as well as its severity in diabetic subjects, particularly in the adult population (Garcia et al., 2015). A number of population studies indicated that not only poorly controlled diabetes metabolic status is associated with increased susceptibility to and severity of inflammatory events in different areas, including periodontal areas, but even an abnormally elevated fasting glucose level may be a predictor for risk of periodontitis (Hong et al., 2016). The increased prevalence of periodontitis within diabetes context is associated with various conditions and factors, from alterations in the immune response, formation and deposition of glycoprotein complexes on the capillaries walls of the gingiva, to altered oxidative status, with modifications in the polymorphonuclear function and hyperlipidemia.

The general part, the actual stage of knowledge, include 7 figures and the personal part 65 figures and 21 tabels.

B. PERSONAL CONSTRUCTIONS

OBJECTIVES

Although the oral cavity is easily accessible to direct clinical examination, and diagnosis of oral disease in systemic context is relatively easy and readily established clinical (based on subjective and objective signs), though certain details of pathological processes escapes usual clinical investigations, being unnoticed. This raises the need to complete clinical examination with laboratory tests that guide us in the diagnosis, etiopathogenesis, prognosis, evolution and individualized therapy management.

Diabetes mellitus (DM) is one of the most prevalent chronic diseases in children, however, it is diagnosed late, or when the child has diabetic ketoacidosis. Diabetes affects children's lives by the need to continuously monitor glucose levels and administer insulin to respect the right balance between food regimen and exercise. Also, diabetes is one of the risk factors in periodontal disease (PD), a bidirectional relationship
between diabetes and BP being recently highlighted by the American Academy of Periodontology (AAP). Moreover, reports of the World Health Organization (WHO) are extremely worrying, indicating that there are currently, worldwide, over 200 million diabetics, with estimated increase their number to 380 million in the next 20 years.

To assess the prevalence of oral periodontal damage in juvenile population with type 1 diabetes (T1D), besides the analysis of genetic factors we investigated the immuno-inflammatory response both, on systemic and local, oral level. All these investigated elements were correlated with clinical and laboratory balance in the study groups, by assessing the clinical indicators of periodontal disease, which expresses the existence and severity of systemic manifestations in the context of diabetes in children and adolescents.

The main objectives of our research focused upon:

1. Determination of the level of interleukins IL-2, IL-4, IL-5, IL-10, IL-18, TNF-α (tumor necrosis factor alpha), IFN-γ (interferon gamma) and IL-1β in the GCF and cell cultures of PBMC (peripheral blood mononuclear cells).

2. Analysis of genomic DNA to assess the gene polymorphisms IL-1A (-889 site), IL-1B (+3954), TNF-α (-308) and TLR4 (896 A/G and 1196 C/T).

CHAPTER I

IV. MATERIALS AND METHODS

IV.1. Clinical and paraclinical evaluation

Evaluation of clinical indicators was achieved by oral examination in the dental office. Thus, PI (plaque index), CI (index scale), IS (bleeding index), PAC (loss of clinical attachment) were correlated with age, the age of diabetes and metabolic control (reflected by values of glycated hemoglobin - HbA1c).

The study included both clinical and laboratory investigations in the 3 categories of patients under study. Thus, the study included a total of 56 children and adolescents aged 6-18 years (age range covering from prepubertal to puberty and juvenile period), included in two large study groups: control group - (M batch) - 24 children (6-18), with general diseases; group of subjects with diabetes mellitus (T1D) (group D) - 32 children (18 girls and 14 boys) aged 6-18 years. Patients in this last cohort were subdivided based on the parameters certifying the degree of metabolic control of diabetes disease in two: group D1 - 17 children with better and moderate diabetes control (HbA1c <8%) and D2 - 15 children and adolescents with poor metabolic control (HbA1c> 8%) of the diabetic disease. In this study there were included only children whose parents or grandparents belonged to the European race (North Caucasians), to reduce genetic heterogeneity within batches.

The investigated samples included:
- serum, for analysis of biochemical parameters of diabetes
- plasmæ for determining the concentration of pro- and anti-inflammatory cytokines synthesized and secreted by plasma cells during the inflammatory response of leukocytes, stored at -70 °C
- total blood collected on heparin (for immunophenotyping of leukocyte populations and subpopulations)
- gingival crevicular fluid (LG-GCF) which has several advantages over blood and saliva, enabling the use of suitable samples from specific sites.

GCF was harvested after bringing patients into the dental office, using the least aggressive method, avoiding mechanical irritation. Paper strips (Periopaper ProFlow Inc., Amityville, NY) were inserted for 30 seconds in the gingival sulcus, interproximal, at clinically relevant areas (redness and local changes of gum consistency). From the harvested gingival fluid, 50 ml PBS specimens for FCM (flow cytometry) have been separated.

IV.2. Determination of cytokines level

The method used for determining the quantities of cytokines both in serum and GCF, as well as to investigate the phenotype of the cell, was flow-cytometry. The level of interleukins IL-2, IL-4, IL-5, IL-10, TNF-α (tumor necrosis factor alpha), and IFN-γ (interferon-gamma) were measured by means of a multiplex kit: CBA (cytometric bead array) for flow cytometry (FCM).
For determining the amount of IL-18 ELISA immunenzimatic method method was used. IL-1β levels were investigated by ELISA using an ELISA "Human Interleukin-1β (hIL-1β)" Biosource kit.

In order to obtain PBMC cell cultures, 15 ml of blood in 10% EDTA tubes were collected from each patient by venipuncture, and the cells were isolated by centrifugation in Ficoll-Hypaque density gradient. By FCM different cells can be sorted by size, granularity and expressed specific markers.

**IV.3. Investigation of mononuclear cells phenotype and peripheral granulocytes function**

Investigation of the phenotype and function of granulocytes and mononuclear cells in the periphery was also conducted by FCM, by the marking of cell surface with monoclonal antibodies conjugated to fluorochromes. Data acquisition was obtained on the FACS (FACS Calibur, BD, 2 lasers).

**IV.4. DNA genomic analysis for investigation of gene polymorphism for IL-1A (-889), IL-1B (+3954) and TNF-α (-308)**

Genomic DNA analysis to evaluate IL-1α gene polymorphisms (-889), IL-1β (+ 3954) and TNF-α (-308) was achieved by PCR sequences amplification, digestion with restriction enzymes and EF (electrophoresis) visualization.

**IV.5. DNA genomic analysis for investigation of gene polymorphism for TLR4 896 A/G and 1196 C/T**

For salivary DNA extraction, Genomic DNA Purification Kit Wizard®, Promega was used, according to the manufacturer's specifications. PCR protocol was similarly used in the evaluation of polymorphisms IL-1A gene (at site -889), IL-1B (in site + 3954) and TNF-α (the site -308) and adapted to the technique previously described.

**CHAPTER V. RESULTS**

**V.1. Clinical and paraclinical evaluation in the studied groups**

Our results registered important correlation of the clinical status of periodontal function, both with biological parameters of the diabetes and imunoinflammatory response of the host. Age analysis of patients in the study highlights the inflammatory gum impairment at ages significantly lower than previously estimated in the literature (p = 0.0048, confidence interval CI 95%), while forms of chronic marginal superficial periodontitis were observed in patients with diabetes with ages around puberty (Figure 2).

![Fig. 2. The diagram of the age values for the studied groups](image)

It is likely that periodontal changes around this age stage is modulated by the association between the metabolic control of diabetes and hormonal changes during puberty or before.

Evaluation of clinical indicators of inflammatory impairment within periodontal areas: correlated with age, length of diabetes and metabolic control indicate the following conclusions: papillary bleeding index (IS) in patients with DM youth has a value significantly higher (p = 0.00, 90% CI) , ANOVA test displaying significant
differences (p << 0.05, p = 0.0008, 95% CI) between the mean age of diabetes by IS. The high value of the correlation coefficient (r = 0.64) confirms the existence of a direct correlation between the IS and oldness of the diabetes disease but not with patients age (r = -0.21, p = 0.79, 95% CI) in the groups studied.

Moreover, the correlation of HbA1c with clinical parameters of impaired oral function indicates that moderately or poorly controlled diabetes (HbA1c > 8%) is associated with elevated IS, HbA1c level in periodontitis being significantly higher than the values recorded for the other studied groups. PAC correlates well with HbA1c values (Figure 5).

Comparing parameters that indicate the degree of periodontal alteration (PAC, IS) with age of onset of systemic disease led us to the conclusion that the oldness of diabetes (p = 0.03684, F = 5.89, 95% CI) and metabolic control thereof may be indicators in assessing determinants of diabetes as a risk factor in periodontal disease.

**Fig. 5.** Mean IS levels in the controls and T1D groups, with good- or poor diabetes-control

Mean IS in poorly controlled T1D is of 2.964 (Figure 5) being almost twice higher than in the control group (IS =1.56) and 1.75 times higher than in T1D better controlled (IS = 1.56). These results can be explained by increased activity of collagenase and vascular changes that increase gingival bleeding in diabetes, thickening of the basement membrane of small vessels within the gum.

Distribution of PAC values show that the elevated levels (between 1.5 - 4 mm) are found in the group of subjects with poorly controlled diabetes (Figure 6), having the highest average value (PAC = 1.053 mm), 2.7 times than the control group, and 3.25 times higher than in T1D young subjects with good metabolic control.

**Fig. 6.** Mean levels of PAC in M and D1 batches (good metabolic control) and D2 (poor metabolic control)

V.2. Determination of local and systemic cytokines level

The gingival fluid (GCF) is a serum transudate that potentially transforms into inflammatory exudate, which bathes the gingival sulcus and follows an osmotic gradient in the local tissues. Our study aimed to investigate the inflammatory mediators of cytokine type in the GCF and peripheral blood, in children and adolescents with T1D, correlated with clinical characteristics of impaired oral and laboratory parameters which reveals metabolic control in these patients.

Of the ample range of inflammatory mediators identified in the oral fluids, cytokines attracted our attention, the most likely involved alike, both in alterations (immunoinflammatory mediated response) and in periodontal tissue repair. Thus, it can be seen that in the plasma of patients there is IFN-γ at a level that is
moderately or significantly increased compared to that of control in GCF, significantly higher in patients of group 1 (Figure 7 and Figure 10).

This can be explained by alterations in the oral microenvironment determining significantly higher glucose and urea GCF values, in patients with diabetes (data recorded by analysis laboratory parameters in the GCF), creating favorable bacterial changes with altering the host's immune response to periodontal pathogens.

**IL-10** has an average level of secretion in blood (Figure 8 and 11), decreased in the group with severe periodontal breakdown (GCF values being inconsistent in the studied groups), being possible that exactly this reduction in secretion of IL-10 to play a role in shifting toward alteration of the oral tissues within juvenile diabetes population.

These results indicate that microbiological interfering involves considerable body efforts, resulting in a significant secretion of IFN-γ. All these suggest that measurement of the fluid gingival levels of interferon, can be an important parameter in assessing T1D progress toward inflammatory or destructive periodontal alterations.

The level of **IL-2** is very small both in the periphery and local (GCF) in patients from group 1 and 3 (Figure 9 and 12), which suggests the possible existence of a local factor that blocks secretion by lymphocytes and macrophages of this T-cell proliferation factor, especially in diabetic patients with periodontal alteration.
Secretion of IL-4 and IL-5 recorded relatively low levels of expression in both systemic and local level for all groups of patients, larger in the case of IL-5 (Figure 16, Figure 17, Figure 19 and Figure 20). The pattern of distribution is quite irregular in the three groups, the values of IL-4 in GCF of group 1 of subjects being significantly higher than those of control.

Furthermore, comparison of clinical parameters with local and peripheral cytokines level reveals a significant direct correlation of IFN-γ values from GCF with IS, and significant inverse correlation with those from serum. IS either correlated well with the values of TNF-α in the gingival fluid but not in serum.

The different secretion amount between groups of diabetic patients with or without periodontal breakdown are more apparent in the periphery, either for TNF-α (Figure 15 and Figure 18), IL-4, IL-5, and IL-2 (the latter with the absolute values of secretion lower than with IL-4 and IL-5). IL-4 in the gingival fluid has a direct moderate correlation with the IS values. IL-5 values in GCF were significantly associated with IS by means of a direct correlation (p = 0.0154, r = 0.526), but with reverse correlation in serum.
The results of the phenotype investigation for the peripheral leukocyte population involved in mediating the immunoinflammatory response, reveals primarily a significantly higher number of T lymphocytes in the group 1 versus group 2, aspect that correlates directly with significantly increasing T helper lymphocytes number in subjects with severe periodontal impairment (Figure 29, 30 and 31).

CD4⁺ T lymphocytes express a reduced level of Fas molecule (CD95⁺), without statistically significant differences in the groups under study. Of the total inflammatory T cells, only a small percentage was in apoptosis, suggesting lack of statistical significant differences in the studied groups.

MFI (mean fluorescence intensity) analysis in terms of intensity of expression of the molecule CD95⁺ are to support the previously uppermentioned data. Furthermore, the intensity level of expression of the coreceptor molecule for Th lymphocytes is significantly different from group 1 compared to groups 2 and 3, with no significant difference in the level of expression of the same molecules in cytotoxic T lymphocytes (Figure 32, 33, 34 and 35). Analysis of the graphs showing the absolute values of the peripheral T cell populations allows us to point out that the expression of activation marker DR/DP/DQ on the surface of T lymphocytes recorded a bimodal response.
This bimodal response has important higher significance in the CD4+ population of patients with severe periodontal breakdown compared with those in the same group (1) but with moderately impaired periodontium (gingivitis) (Figure 36), while at the level of cytotoxic lymphocytes, the relationship is reversed (Figure 40).

Moreover, CD69+, another marker selectively activating lymphocyte populations, recorded in CD4+ T cells similar patterns as to DR/DP/DQ, but at significantly lower levels (Figure 37). Considering the CD8+ population, this marker is best represented in the control group, with statistically significant differences in absolute values (much increased) compared to groups 1 and 2 (Figure 41).
Fig. 37. CD4+ population cells that express activation markers and local proliferation (CD69+) (p=0.00005)

Fig. 38. CD4+ population cells that express activation markers and local proliferation (CD25+) (p=0.0929)

On the other hand, CD25+ is better represented in the CD4+ T cells (Figure 38), than at the level of CD8+ T cells (values of 0, not shown).

Fig. 39. CD8+ population cells that express activation markers and local proliferation (CD95+) (p=0.0019)

Fig. 40. CD8+ population cells that express activation markers and local proliferation (DR/DP/DQ+) (p=0.0000005)

Fig. 41. CD8+ population cells that express activation markers and local proliferation (CD69+) (p=0.3707)

Analysis of the activation marker DR/DP/DQ in the population of granulocytes and monocytes of peripheral blood reveals responses with different expression levels in the two leukocyte subpopulations. Thus, the number of monocytes of mononuclear cell type expressing on their surface this marker is significantly greater than that of granulocytes in the periphery (Figure 42). Moreover, the pattern of expression of this marker at monocyte level recorded statistically significantly higher values in the control group compared to the diabetes group without periodontal breakdown, but more prominent especially when compared with patients with periodontitis (Figure 43).
The outcomes of our comparative research upon the three studied groups suggest that diabetes associated with periodontal tissue impairment (gingival inflammation or periodontitis) is an immune imbalance orchestrated mainly by T lymphocytes. One possible explanation for these changes is the fact that CD4+ T lymphocytes stimulated Th1-type inflammation in the gingival young patients with T1D produce IFN-γ-type cytokines, which probably inhibit the production and activation of Th2 cells, with a subsequent decrease in IL-4, IL-5, IL-10.

Local Th1/Th2 response tilted therefore in favor of Th1 in groups of children and adolescents with T1D associating signs of impaired periodontal tissues. This draws attention to the precautions in interpreting and comparing the results of studies that monitor the same parameters, but applying different technology (FCM, ELISA, hybridization in situ), or different biological samples (blood line isolated T cells, gingival tissue) and are generating controversy as is the case of the predominance of the immune response in the periphery in periodontitis (but not associated with diabetic disease) and was reported as being either Th1, or Th2.

For group 1 patients, the lowest levels of IFN-γ in GCF were recorded in diabetic patients with clinical signs of periodontitis, suggesting local Th1 phenotype depression in these conditions (unlike patients from the same batch but with minimum alteration of gingival inflammation type, and developing a local Th1 phenotype).
IL-1β INVESTIGATION

The levels of the interleukin-1β in GCF are shown in figure 44. The patients in the two main groups (Control and D, the latter subdivided into D1 and D2) have been grouped in the present substudy into the nine categories based on periodontal and systemic health. According to our data presented in tables and plotted, systemic unaffected teenagers with the mildest periodontal alter (ie gingivitis) have recorded lowest level of IL-1β in GCF, respectively 109.364 ng/ml. IL-1β values increase significantly in this lot, along with disease severity, with an increment of about 2-fold in patients with aggressive periodontitis (211.16 vs. 109.36 ng / ml).

![Figure 44](image)

**Fig. 44.** Comparative analysis of mean IL-1β levels depending on systemic and periodontal alter

A significant increase in interleukin 1β has been found associated to T1D. The values of this biochemical mediator were found on a range stretched from 347.889ng/ml in T1D subjects with gingivitis and tight metabolic control, to 1274.667 ng/ml in aggressive periodontitis T1D subjects with poorly metabolic control, thus registering a significant 3.21 times elevation of the GCF cytokine secretion in good controlled T1D vs. witness (378.804 vs. 117.847 ng/ml) and a severe 7.35 times increase of IL-1β between poorly controlled T1D and systemically unaffected individuals (866.733 vs. 117.847).

Comparative analysis of the IL-1β levels between diabetic diagnostic categories (A - gingivitis; B - superficial chronic marginal periodontitis, C - aggressive periodontitis) reveals:

- ♦ in diabetic subjects with good metabolic control, values of 1.75 times higher in patients with aggressive periodontitis than those with gingivitis (610.667 vs. 347.889ng/ml, p = 0.0252);
- ♦ in T1D children and adolescents with poorly controlled metabolism average values are growing by 1.42 times in chronic marginal periodontitis than those with gingivitis (1034.933 vs. 727.96 ng/ml, p = 0.008) and 1.75 times in aggressive periodontitis than those with gingivitis (1274.667 vs. 727.963ng / ml, p = 0.000026).

Much more relevant are comparisons by diagnosis of IL-1β values between diabetic subjects and systemic healthy subjects (controls). * Among children with gingivitis and better T1D control and healthy children with gingivitis there is an systemic IL-1β augmentation of 3.18 times (347.889 vs. 109.364 ng/ml, p = 0.000145); * In aggressive periodontitis subjects with tightly controlled T1D, IL-1β values increased of 2.89 times compared to its value in children with severely affected periodontum but systemically un affected (610.667 vs. 211.167, p = 0.001939); * More important significant differences were recorded between IL-1β in subjects with poorly metabolic controlled T1D and gingivitis, compared to the systemic healthy gingivitis (727.963 vs. 109.364 ng/ml), with an increase of 6.65 times (p = 0.000018); * A similar pattern was recorded in children with poorly controlled T1D and aggressive periodontitis (1274.667 ng/ml) compared to the same periodontal alter degree of non-diabetic children (211.167 ng/ml) registering a growth of 6.03 (p = 0.000026).
For IL-18 determination, as already mentioned, the selected patients were included in eight groups, namely controls (healthy, with mild, medium or severe gingivitis) and diabetes (non-periodontaly affected, with mild, medium or severe gingivitis). The purpose of the study was to establish positive correlations between the degree of gingival inflammation and the concentration of IL-18 in gingival crevicular fluid, in children and adolescents with T1D.

The analysis of the studied clinical indicators groups is reflected in Table 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>T1D</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP (average±SD)</td>
<td>Gr. 1 (GI=0)</td>
<td>Gr. 5 (GI=0)</td>
<td>Gr 1 vs 5, p=0.431</td>
</tr>
<tr>
<td></td>
<td>Gr. 2 (GI=1)</td>
<td>Gr. 6 (GI=1)</td>
<td>Gr 2 vs 6, p=0.199</td>
</tr>
<tr>
<td></td>
<td>Gr. 3 (GI=2)</td>
<td>Gr. 7 (GI=2)</td>
<td>Gr 3 vs 7, p=0.075</td>
</tr>
<tr>
<td></td>
<td>Gr. 4 (GI=3)</td>
<td>Gr. 8 (GI=3)</td>
<td>Gr 4 vs 8, p=0.802</td>
</tr>
<tr>
<td>IG (average±SD)</td>
<td>Gr. 1 (GI=0)</td>
<td>Gr. 5 (GI=0)</td>
<td>Gr 1 vs 5, p=0.445</td>
</tr>
<tr>
<td></td>
<td>Gr. 2 (GI=1)</td>
<td>Gr. 6 (GI=1)</td>
<td>Gr 2 vs 6, p=0.218</td>
</tr>
<tr>
<td></td>
<td>Gr. 3 (GI=2)</td>
<td>Gr. 7 (GI=2)</td>
<td>Gr 3 vs 7, p=0.025*</td>
</tr>
<tr>
<td></td>
<td>Gr. 4 (GI=3)</td>
<td>Gr. 8 (GI=3)</td>
<td>Gr 4 vs 8, p=0.023*</td>
</tr>
<tr>
<td>IS (average±SD)</td>
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<td>Gr. 5 (GI=0)</td>
<td>Gr 1 vs 5, p=0.073</td>
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<td>Gr. 2 (GI=1)</td>
<td>Gr. 6 (GI=1)</td>
<td>Gr 2 vs 6, p=0.195</td>
</tr>
<tr>
<td></td>
<td>Gr. 3 (GI=2)</td>
<td>Gr. 7 (GI=2)</td>
<td>Gr 3 vs 7, p&lt;0.01*</td>
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<td>Gr. 4 (GI=3)</td>
<td>Gr. 8 (GI=3)</td>
<td>Gr 4 vs 8, p=0.025*</td>
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<tr>
<td>PD &lt; 3mm (average±SD)</td>
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<td>Gr 2 vs 6, p=0.719</td>
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<td>Gr. 7 (GI=2)</td>
<td>Gr 3 vs 7, p=0.151</td>
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<td>Gr. 8 (GI=3)</td>
<td>Gr 4 vs 8, p=0.397</td>
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<td>CAL &lt; 2mm (average±SD)</td>
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<td>Gr. 5 (GI=0)</td>
<td>Gr 1 vs 5, p=1.000</td>
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<td>Gr. 4 (GI=3)</td>
<td>Gr. 8 (GI=3)</td>
<td>Gr 4 vs 8, p=0.183</td>
</tr>
</tbody>
</table>

*p<0.05, statistic significant

In all the studied groups, IL-18 were higher at the incisors level.

It is to be mentioned that based on clinical indicators (PD and CAL), no cases of chronic marginal periodontitits or aggressive periodontitis have been identified, nor any attachment loss greater than 2 mm or amount of PD> 3 mm being recorded.

Also, there were no statistically significant differences between clinical signs as PI, PD and CAL between the corresponding control groups and the T1D groups, but the differences are slightly increased between control and T1D groups for IG and IS. IL-18 concentration in the GCF increased gradually with the progression of inflammation at the periodontal level and was strongly associated with the presence of diabetes (mean values of IL-18 levels in GCF showed values about 2.5 times higher in diabetic children compared to controls).

Our results suggest that periodontal degradation may occur very early in diabetic children, inflammation monitored by IL-18 in GCF being more obvious and earlier detectable than using clinical indicators thereof (IG, IS, PD and CAL). Statistical differences in this regard shows that, while average values of clinical indices of periodontal disease, ie IP, PD, CAL, between non-diabetics and T1D showed no significant differences (whereas slightly elevated for IG and IS), when considering IL-18, the differences are significantly marked between the two groups (control and T1D).

Thereby, this suggests that the changes at the preclinical level are triggered (and can be evaluated) before the clinical signs are positive (and may be subjective), determination of IL-18 in GCF being thus appropriate for an early and accurate diagnosis of periodontal injury in juvenile diabetics.
Moreover, IL-18 may be a prognosis marker of periodontal inflammation of diabetic patients when clinical changes have not been installed yet, the values of IL-18 in the group of children with T1D and without clinical expression of the periodontal injury (IG = 0) recording higher levels than those in the group with mild to moderate gingivitis (GI = 2; IG = 3) in the control group (I5 = 20.6 ± 1.7 pg/ml; M5 = 14.2 ± 1.4 pg/ml + I2 = 17.4 vs. 0.9 pg/ml; M2 = 12.5 ± 0.3 pg/ml respectively I3 = 19.5 ± 0.8 pg/ml; M3 = 12.8 ± 0.3 pg/ml).

Our study clearly revealed increased IL-18 concentrations in GCF proportionaly to the progression of the inflammation from gingival to periodontal level, IL-18 values increasing with IG and IS, both in T1D and control group, consistent with studies performed on adult T1D subjects. In addition, this increase in IL-18 in GCF was strongly associated with the presence of diabetes metabolic disorder in children, IL-18 values being much higher in T1D versus control group, but also upon corresponding groups (T1D versus control).

These results could not be compared with other literature data as to our knowledge our research being the first study that assesses GCF IL-18 in children with T1D.

In conclusion, the cytokine response in young T1D patients can be considered an important modulator of the periodontal breakdown, although the extent to which this could be a relevant factor in determining its progression is still a matter of debate.

V.3. Investigation of mononuclear cell phenotype and function of peripheral granulocytes

Research on T1D children have shown that diabetes modulate the expression of cytokines IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ in the GCF as well as the phenotype of peripheral cells involved in the immune response.

Phenotyping mononuclear cells cultures PBMC obtained by separation in density gradient from venous blood collected from all groups of children and adolescents in the study, was achieved both in cell culture for 24 and 48 hours after stimulation with bacterial lipopolysaccharide - LPS E. Coli. It is known that LPS is a common virulence factor of the periopathogenic bacteria, able to penetrate the periodontal tissues with subsequent interaction in the immune and non-immune cells of the host. This interaction induces cell activation, with subsequent release of inflammatory mediators (cytokines, chemokines), responsible, at least in part, by the destruction of the periodontal tissues.

![Fig. 45. Mean CD3+ T lymphocytes in the studied groups](image)

Statistical analysis of the results of individual PBMC cell populations in cultures, in the studied groups, are shown in Figures 45, 46 and 47.

Our study aimed to analyze the impact of bacterial endotoxin on the phenotype and function of peripheral mononuclear cells (PBMC), identifying the signaling pathways activated by bacterial antigen, highlighting the intracellular proteins modulated particularly in macrophages.

Very likely, the signaling pathways are different when signaling the host tissues is achieved through "live" bacterial periopathogen agent (bacteria itself) and not LPS-major component of the outer membrane of gram negative bacteria, known as the ligand to the cellular receptors, mediating the effects of the infective event.

It was observed that not only the production of mediators by the cells is different between the groups under study, but also MFI (mean fluorescence intensity) indicating cell surface expression of the molecule of interest.
(CD14 on monocytes examined by us), especially when comparing its average value between LPS-stimulated vs. unstimulated cultures.

Data recorded by flow cytometry suggests that monocytes and macrophages can be included in the PD development mechanism and provide scientific basis for the management of oral hygiene to prevent the installation of inflammatory periodontal lesions.

Of receptors that mediate the inflammatory response to the action of the bacterial wall components of gram negative bacteria, TLR2 and TLR4 ("toll like receptor" family members) are distinguished (Veloso et al., 2011), with significant role in the induction of cytokine production in macrophages. Our study indicates large IL-6 levels in stimulated cultures, the highest values being recorded in young T1D subjects, consistently more elevated in culture after 48 hours compared to that of 24 hours, suggesting the existence in the medium of a continuous cytokine production stimulating factor.

Moreover, the secretion of large amounts of IL-6 in LPS-stimulated cultures suggests LPS E. Coli actions might be mediated by TLR4 signaling receptor (knowing that this cytokine production is preferentially induced by TLR4 receptor signaling). Of course, it would be interesting to investigate whether the bacteria live and the products of bacterial surface (such as LPS) induces quantitative (signal strength) or qualitative (the panel of cytokines secreted) changes of the biological signal, induced at the macrophages level by differential TLR receptors or interfaces activation involved in pathogeny.

The recorded data indicates the lack of signaling pathways involved in the secretion of certain cytokines, with the registration of very low or undetectable levels of cytokines such as IL-2, IL-4, IL-10 in stimulated (or unstimulated) cells in our groups of investigation.

The levels of IL-6 and TNF-α, however, are strongly influenced by the stimulation of cell cultures with LPS, this fingerprint being relevant in both, diabetics and non-diabetics, although with different magnitudes.

Thus, monocytes response to antigenic stimulus action recorded different amplitude in children with T1D compared to control, the most significant differences being recorded between the average in the aggressive
periodontitis diabetic group and mean value in children without systemic damage. Analyzing the results, one can say that in addition to exploring mononuclear cells in order to fully explain susceptibility to PD, it should be paying attention to other cell lines (epithelial, fibroblasts or endothelial), knowing the fact that they have the ability to interact with bacterial components through TLR, with consequent production of cytokines (Chen et al., 2013).

The study of the immuno-inflammatory response dental plaque-induced in oral areas and expression of the signaling mechanisms induced by the microbial agent in the periphery, by determining the level of cytokines in PBMC cultures, stimulated or unstimulated, in groups of patients with and without impaired systemic impairment, suggests that the major source of pro-inflammatory mediators at this level are the monocytes.

Furthermore, consecutive stimulation with endotoxin these cells express a distinct phenotype, with preferential hypersecretion of certain cytokines, feature that at this time cannot be distinguished as intrinsic or acquired due to diabetes metabolic induced-modifications. Data recorded by flow cytometry suggests that monocytes and macrophages can be included in the development of periodontal disease mechanism and provide scientific basis for the management of oral hygiene to prevent the installation of inflammatory periodontal lesions.

The results recorded in the investigated groups, are consistent with the concept that the cytokine response in young patients with T1D can be considered an important modulator factor in periodontal alterations, but without knowing the extent to which it could be a relevant factor in assessing its progression.

V.4. Genomic DNA analysis to evaluate IL-1A gene polymorphisms (-889), IL-1B (+3954) and TNF-α (-308)

The results of the genotyping for IL-1A reveals that patients 2, 3, 4, 5, 6, 8, 14, 18, and 19 are homozygous for allele 1 (produced by digestion of the 83 pb), and the patient 7, 9, 10, 11 12, 13, 15, 16, 17, and 20 are homozygous for allele 2 (produced by digestion of the 99 pb). From the total of 56 investigated patients,
35 patients are homozygous for allele 1, and 21 patients are homozygous for allele 2. Heterozygous individuals have not been identified. No amplification was observed in the control of NTC (Table 11).

The results of the genotyping for IL-1 on the same selected sample, indicates in this case that the patients 2, 4, 5, 6, 8, 10, 14, 17, 18, and 19 are homozygous for allele 1 (produced by digestion of 85 and 97 pb), and patient 7 is homozygous for allele 2 (182 pb digestion product).

Patients 9, 11, 12, 13, 15, 16 and 20 are heterozygous having both alleles (produced by digestion of 85, 97 and 182 pb). From a total of 56 investigated patients, 37 patients are homozygous for allele 1. 16 patients are heterozygous, and 3 patients were genotyped homozygous for allele 2. There was no NTC amplification control; Instead, the protocol worked well for the two DNA control samples (one allele homozygous genotype 1, and the other heterozygous).

Genotyping for TNF-α (sample of 19 patients) showed that the patients 3, 4, 8 and 9 are homozygous for allele 1 (produced by digestion of 20 and 87 pb), and the patient 20 is homozygous for allele 2 (produced by digestion 107 pb). Patients 2, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 are heterozygous having both alleles (produced by digestion of 20, 87 and 107 pb). Among the total 56 investigated patients, 23 patients are homozygous for allele 1, 32 patients are heterozygous and a patient has been genotyped homozygous for allele 2 (Table 11).

Table 11. Statistic genotyping data for the 56 patients (24 Control and 32 T1D)

<table>
<thead>
<tr>
<th>32 T1D patients</th>
<th>24 Control patients (non-diabetics)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1A(-889) Genotype</strong></td>
<td><strong>IL-1B(+3954) Genotype</strong></td>
</tr>
<tr>
<td>Allele 1</td>
<td>Allele 2</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>47%</td>
<td>53%</td>
</tr>
</tbody>
</table>

| **IL-1A(-889) Genotype** | **IL-1B(+3954) Genotype** | **TNF-α(-308) Genotype** |
| Allele 1 | Allele 2 | h | Allele 1 | Allele 2 | Heterozygote | Allele 1 | Allele 2 | Heterozygote |
| 20 | 4 | 0 | 20 | 1 | 3 | 16 | 0 | 8 |
| 83% | 17% | 0% | 84% | 4% | 12% | 67% | 0% | 33% |

With the limitations imposed by the small size of the study group, screening polymorphisms of some cytokines whose secretion is extensively altered in children with T1D, reveals the following:

1. **IL-1A (-889)** heterozygotes have not been found in any control group or children with T1D;
   - Statistical analysis of the percentage of homozygous for Allele 1 (significantly lower in people with diabetes - 47% vs. 83%), indicated in the T1D group higher values in people with minimal periodontal impairment (gingivitis) compared to those with aggressive or marginal chronic periodontitis. The allele 1 of IL-1A is therefore a protective factor against severe periodontal changes installation in juvenile population with T1D.
   - The prevalence of positive genotype of IL-1A (homozygous for the Allele 2) is markedly increased in children with T1D (53% vs. 17%) and was significantly associated with severe periodontal disease (p=0.002).

2. The prevalence of positive genotype for **IL-1B (3954)** (at least 2 allele present in each locus) is significantly increased in the group with T1D compared with non-diabetic (41% vs. 16%);
   - In addition, the prevalence is proportionaly associated with the severity of the degree of PD
   - Among diabetic patients presenting IL-1B Allele 2 (3954), 85% were heterozygous and only 15% homozygous.

3. Analysis of the prevalence of **TNF-α polymorphism (-308)** in people with T1D juvenile indicate a significantly increased percentage of allele 2 (78% vs. 22% in the control group), among them, a remarkable proportion being heterozygous (95%) and only 5% homozygous.
   *Statistical studies of the molecular changes revealed no significant associations of polymorphic allele with clinical stage of periodontal damage (p = 0.125).
V.5. Genomic DNA analysis to evaluate TLR4 gene polymorphisms, meaning uninucleotide substitutions 896 A/G și 1196 C/T

To achieve these genomic analyzes, the amount of DNA measured using Thermo Scientific NanoDrop 2000 spectrophotometer ranged from 12.6 to 182.3 µg/mL. PCR products obtained were analyzed using specialized software Mx Pro, version Mx3005P for real-time PCR (Stratagene company, Agilent Technologies). With these software tools, complemented with the necessary hardware, each sample genotype polymorphisms has been identified (Figure 64, Figure 65). TLR4 gene polymorphisms, 896 A/G and 1196 C/T have not been recorded in a statistically significant proportion nor in the control-, nor in the T1D group.

![Fig. 64. 1196 C/T genotypes in control and T1D groups](image1)

![Fig. 65. 896 A/G genotypes in control and T1D groups](image2)

CONCLUDING REMARKS

- Children and adolescents with T1D are at increased risk of developing periodontal disease, a direct correlation being found either with the degree of the metabolic control of the systemic alteration, the phenotype of mononuclear cells secreting proinflammatory markers and the rate of cytokines secretion at the peripheral and local oral level. This risk can be exacerbated when combining molecular variations in IL-1α/IL-1β and TNF-α genotype.

- The outcomes of our comparative study upon the three groups suggest that diabetes associated with periodontal breakdown (gingival inflammation or periodontitis) is an immune imbalance orchestrated mainly by T lymphocytes. One possible explanation for these changes is the fact that CD4+ T lymphocytes stimulate Th1-type inflammation in the gingival young patients with T1D to release IFN-γ-type cytokines, which probably inhibit the production and activation of Th2 cells, with a subsequent decrease in IL-4, IL-5, IL-10. Furthermore, these data reinforce the concept that periodontal damage may occur very early in diabetes child, inflammation monitored by IL-18 in GCF being more obvious and earlier detected than using clinical indicators thereof (IG, IS, PD and CAL).

- Thereby, one can suggest that the changes at the preclinical level are triggered (and can be evaluated) before the clinical evidences (which are subjective), determining GCF levels of IL-18 being appropriate to establish an early and proper diagnosis of periodontal injury for juvenile diabetes subjects.

- The study results and statistical analysis percentage, albeit with limits of rigor imposed by the relatively small size of the investigated sample, reveals important differences in genotypic distribution of IL-1α (−889), IL-1β (+3954) and wild alleles, with significantly changes along diabetic children (with poor or good metabolic control as well) and those without systemic damage. Moreover, there is a direct correlation between positive genotype prevalence and severity of periodontal damage.

- The presence of a composite specific IL-1α/IL-1β genotype (consisting in the presence of allele 2 as in the case of IL-1α and IL-1β), was detected in a small number of patients, so that it can confirm the existence of a relationship between it and the susceptibility to periodontal disease of T1D children and adolescents.

- The statistics of TNF-α polymorphism analysis (−308) among juvenile T1D also suggest correlation of the presence of abnormal allele more frequently in the diabetic population, without significant association with the severity of oral impairment.
Regarding TLR4 gene polymorphisms, 896 A/G and 1196 C/T, they are not encountered in a statistically significant proportion nor in the control group, nor for T1D group.

The cytokine response in young patients with T1D can be considered an important modulating factor of periodontal damage expression, but without knowing the extent to which it could be a relevant factor in assessing their progression.

The results of molecular analysis correlates significantly with the level of cytokines secreted by PBMC culture supernatants from patients and controls, revealing a direct correlation between IL-1β polymorphisms and cytokine secretion in cell culture.

Corroborating clinical status with laboratory findings, statistical processing and interpretation of results allows us to identify an immune imbalance and draw conclusions that represent real alarm signals, both in terms of managing disorders in the dental office (a challenge even for the most skilled clinicians) and the need to establish an interdisciplinary medical community to raise awareness of the interrelation diabetes-periodontal disease.

**PERSPECTIVE OPENED BY THE THESIS**

- Screening of IL-1α, IL-1β and TNF-α polymorphisms in assessing predisposition to develop periodontal disease in children and adolescents with T1D is only a preliminary study of molecular variance analysis that we intend to expand in the juvenile diabetes population living in Moldavia counties.

- Given that diabetes is a modifier factor that can not be removed but which may however be controlled, we assume that, very probably, therapeutic manipulation of the immune system by targeted modulation of specific cytokines (TNF-α, IFNγ, IL-10) can be a prerequisite in the T1D children and adolescents standard care.

- These preliminary outcomes are quite promising in determining the potential of periodontal lesions installation in children with T1D, extending the batches being mandatory in order to determine the impact of genes polymorphisms involved in risk assessment.

* In the present summary, figures are shown selective, respecting the correspondence from the thesis.

**SELECTIVE BIBLIOGRAPHY**


